ENVIRONMENTAL HEALTH PERSPECTIVES
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http://dx.doi.org/10.1289/ehp.1409586

Received: 11 December 2014
Accepted: 3 June 2015
Advance Publication: 5 June 2015

Editor's Note: An error was found in this article after acceptance. The correction is included before the original manuscript. The error will be corrected in the final version of the article.

Correction

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Information that was provided in the Advance Publication regarding NOAEL values defined for DEP and triclosan has been revised to indicate the individual studies on which the NOAELs were based, including Brown et al. (1978), Moody and Reddy (1978), and Oishi and Hiraga (1980) for DEP; and Goldsmith and Craig (1983) and Rodriguez and Sanchez (2010) for triclosan.

Environ Health Perspect DOI: 10.1289/ehp.1409586
Advance Publication: Not Copyedited

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Running title: Paired serum and urine biomarkers in rats

Acknowledgments: We thank Ella Samandar, Jim Preau, Xiaoliu Zhou, Josh Kramer and Lily Jia at the CDC for technical assistance in measuring the biomarker serum and urine concentrations. We thank Karen Miller, Huntington Breast Cancer Action Coalition and Laura Weinberg, Great Neck Breast Cancer Coalition for their input on the project. This research is supported by NIEHS/NCI: 5U01ES019459.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC.

Competing financial interests: The authors declare that they do not have any competing financial interests.

Abstract

Background/Objective: This first of its kind proof of concept rodent study examines the relationship between oral doses of three widely used personal care product ingredients (diethyl phthalate (DEP), methyl paraben (MPB), triclosan) and urine and serum concentrations of their respective biomarkers.

Methods: Using female Sprague-Dawley rats, we carried out two rounds of experiments with oral gavage doses selected in reference to EPA NOAEL: 1735 (DEP), 1050 (MPB), 50 (triclosan) mg/kg/day. Administered doses ranged from 0.005-173 mg/kg/day, 10-100,000 times below NOAEL for each chemical. Controls for MBP and triclosan experiments were animals treated with olive oil (the vehicle) only; controls for DEP serum experiments were animals treated with the lowest MBP and triclosan doses. Doses were administered for five days with five rats in each treatment group. Urine and blood serum, collected on the last day of exposure, were analyzed for biomarkers. Relationships between oral dose and biomarker concentrations were assessed using linear regression.

Results: Biomarkers were detected in all control urine samples at parts-per-billion levels suggesting a low endemic environmental exposure of the three chemicals that could not be controlled even with all precaution measures undertaken. Among exposed animals, urinary concentrations of all three biomarkers were orders of magnitude higher than those in serum. A consistently positive linear relationship between oral dose and urinary concentration was observed ($R^2 \geq 0.80$); the relationship was inconsistent in serum.

Conclusions: Our study highlights the importance of careful consideration of the oral dose used in animal experiments and provides useful information in selecting doses for future studies.

Introduction

Exposure to environmental chemicals, including phthalates and phenols such as parabens and triclosan, is ubiquitous within the US general population (CDC 2014). There is growing interest in the possible adverse human health outcomes associated with exposure to these chemicals (Diamanti-Kandarakis et al. 2009).

Phthalates are used in a wide range of consumer goods (CDC 2014). Low molecular weight phthalates (e.g., diethyl phthalate [DEP]) are often found in personal care products (e.g., fragrances, shampoo, cosmetics). In rats, DEP is hydrolyzed to its monoester metabolite, monoethyl phthalate (MEP) (Albro and Moore 1974); in humans, metabolism is assumed to be similar (ATSDR 1995). Elimination half-lives of DEP and MEP in mammals have not been experimentally defined, but are believed to be a few hours (Calafat and McKee 2006). Therefore, MEP has been used as a biomarker of recent exposure to DEP. Several health effects have been associated with higher urinary concentrations of MEP, including: adverse male reproductive outcomes (Duty et al. 2003;Hauser et al. 2007;Jonsson et al. 2005;Swan et al. 2005); altered neonatal behavior and neurobehavioral development (Engel et al. 2009;Engel et al. 2010;Wolff et al. 2008); and increased breast cancer risk (Lopez-Carrillo et al. 2010).

Parabens are commonly used as preservatives in personal care products, cosmetics, pharmaceuticals and even in the processing of foods and beverages (Golden et al. 2005). Most Americans are exposed to various parabens, including methyl paraben (MPB) (CDC 2014). Because of their potential estrogen activity, it has been suggested that parabens may play a role in breast cancer, albeit orders of magnitude lower than that of endogenous estrogens (Darbre and Harvey 2008;Golden et al. 2005); however strong epidemiologic evidence is lacking (McGrath 2003;Mirick et al. 2002). Parabens are hydrolyzed to *p*-hydroxybenzoic acid, which can be conjugated and excreted in urine. *p*-Hydroxybenzoic acid or its conjugates are non-specific metabolites of all parabens (Ye et al. 2006). In contrast, the concentrations of total (free plus conjugated) urinary species of the parent parabens are considered valid human exposure biomarkers (Ye et al. 2006) and have been used as measures of environmental exposure to these chemicals in epidemiologic studies (Mervish et al. 2014;Wolff et al. 2010;Wolff et al. 2014).

Triclosan is a commonly used antimicrobial in personal care and household products ranging from toothpaste, deodorant and hand soap to cutting boards and textiles (National Library of Medicine 2015). Public interest has been steadily increasing about the ubiquitous exposure sources to this chemical. The hormonal activity of triclosan has not been clearly established, due to the conflicting results from different investigations. There is evidence of weak estrogenic (Chen et al. 2007;Svobodova et al. 2009) and androgenic activity (Chen et al. 2007), estrogen receptor antagonism (Ahn et al. 2008), and anti-androgenic properties (Chen et al. 2007). The excretion half-life of triclosan has been estimated as 11 hours for urine and 21 hours for plasma (Sandborgh-Englund et al. 2006). Triclosan excreted in urine is mainly in its conjugated form while the percentage of free triclosan is higher in plasma (Sandborgh-Englund et al. 2006). Urinary concentrations of triclosan (conjugated plus free species) can be used as an exposure biomarker (Calafat et al. 2008).

Given the variability in their bioactivities, the understanding of dose-response relationship is fundamental and essential for studying the potential biologic or health effects of these chemicals. Furthermore, the dose-response relationship may depart from linearity at low doses (Vandenberg et al. 2012) making dose-extrapolation difficult and potentially unreliable. Thus, for evaluating risk or biological effects of environmental chemicals, it is critical to employ doses in animal experiments that are comparable to the human experience. However, the dose range of DEP, MPB and triclosan used in animal studies has been wide and often orders of magnitude higher than humans are likely to encounter (Shiraishi et al. 2006;Stoker et al. 2010;Vo et al. 2010). Based on growing evidence suggesting low-dose health effects, there is an urgent need for studies utilizing doses in the range of typical human exposures (Birnbaum 2012;Casals-Casas and Desvergne 2011).

The objective of this study was to investigate the relationship of oral doses of three widely used personal care product ingredients (DEP, MPB, and triclosan) in rats with the resulting urinary and serum biomarker concentrations. We relied on a traditional rat model, commonly used in toxicology and risk assessment research. The rat has been used extensively for developmental and reproductive physiology and endocrinology research, and has been more thoroughly characterized in these research fields than other species; likewise for identifying likely human carcinogens (Gray, Jr. et al. 2004;Maltoni et al. 1999). Although these experiments are part of a larger study examining personal care product ingredients and breast cancer risk, the results of our study will provide a foundation for future rodent-based health risk assessment studies for human exposure to these chemicals.

Methods

Materials and standards

Diethyl phthalate (CAS # 84-66-2, lot # STBB0862V, 99% purity), methyl paraben (CAS # 99-76-3, lot # BCBG0852V, 99% purity) and triclosan (CAS # 3380-34-5, lot # 1412854V, 97% purity) were supplied by Sigma Aldrich (Milan, Italy). Olive Oil (Montalbano Agricola Alimentare Toscana, lot # 111275, Florence, Italy) was used as vehicle to prepare all dosing solutions. During the experiment, each compound was stored at room temperature (20 °C) and in the dark. The solutions were prepared the first day of treatment for the whole duration of the experiment (5 days) and were continuously stirred throughout the study; the stability of the solutions was confirmed by gas chromatography-mass spectrometry (GC/MS) (Neotron Laboratory, Modena, Italy).

Experimental animals

All animal study procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC), following the principles of the Good Laboratory Practices and Standard Operating Procedure of the Ramazzini Institute (RI) facility. The protocol was also approved by the Mount Sinai IACUC. The animals were treated humanely and with regard for alleviation of suffering. This study was designed as a proof of concept experiment to determine oral doses to be used in a larger investigation focusing on the potential effects of these chemicals on mammary tissue gene expression; therefore, only female rats Sprague-Dawley (SD) rats were used for this study. This strain belongs to the colony used for over 40 years in the laboratory of the Cesare Maltoni Cancer Research Centre of the RI facility. The animals were distributed into ten groups and randomized in order to minimize the number of animals from each litter in the same group. Rats were identified by ear punch according to the Jackson Laboratory system. Throughout the treatment period prior to urine and blood collection, animals were housed in standard polycarbonate cages (41x25x15cm) with two or three rats per cage and with a stainless steel wire top and a shallow layer of white wood shavings as bedding. Cages were identified by a card indicating the experiment, the group and the experimental number/pedigree number of each animal. During each experiment, all animals were kept in a single room at 23±3°C and at 40-60% relative humidity. Lighting was natural or artificial to maintain the light/dark cycle of 12 hours. Before starting the treatment, animals were weighed and dosed based on the average weight of the experimental group (mg/kg b.w.). Rat feed (Dr. Piccioni Laboratory, Milan, Italy) and tap water were provided *ad libitum*. Each lot of feed and tap water was periodically analyzed for biologicals (bacteria) and chemicals (mycotoxins, pesticides, arsenic, lead, mercury, selenium); they were not tested for DEP, triclosan or MPB. The experimental design is shown in Table 1. To reduce the possibility of DEP, triclosan or MPB contamination, all cages, containers and syringes used during the experiments were washed without using detergent; hot water over an extended period of time was used for cleaning.

Chemical treatment

Two rounds of experiments (Table 1) were performed to identify the oral gavage administered dose of each chemical that resulted in urinary concentrations of the corresponding biomarker within the ranges reported in the US National Health and Nutrition Examination Survey (NHANES) (CDC 2014). For each round, three doses were selected for each chemical based on its "no observed adverse effect level" (NOAEL), i.e. 1735, 1050, and 50 mg/Kg/day for DEP, MPB, and triclosan (U.S. EPA 2002;U.S. EPA 2005;U.S. EPA 2008).

Female SD rats were treated daily for 5 days by oral gavage. Each treatment group consisted of 5 rats including a control group with rats treated with olive oil only. A total of 50 rats, with 10 experimental groups (3 chemicals x 3 doses + 1 control) were involved in each experimental round. For both experimental rounds, the dosing day was designated as day 0, urine and serum samples were collected on day 6. The first experiment was carried out with rats at 16 weeks of age; the selected testing doses were NOAEL/10, NOAEL/100 and NOAEL/200 for each of the 3 target chemicals. The second experiment was carried out with rats at 27 weeks of age; the testing doses were NOAEL/200, NOAEL/10,000 and NOAEL/100,000 for DEP and MBP, and NOAEL/200, NOAEL/1,000 and NOAEL/10,000 for triclosan.

To minimize external contamination, the olive oil and chemicals were stored in glass containers and administered using 5 ml glass syringes. DEP, triclosan and MPB were not detected using GC/MS in the olive oil used as vehicle at Neotron Laboratory, Modena, Italy (www.neotron.it). Biological samples were collected in polypropylene vials. At the end of the experiment and after the urine collection (day 6), each rat was sacrificed by CO_2 inhalation.

Urine collection

The morning after completion of treatment (day 5), rats were moved from their experimental cages to metabolic cages (TECNIPLAST S.p.A., Italy) where each rat was individually housed for 24 hours without food supply, but with water ad libitum. Metabolic cages are specifically designed to prevent fecal contamination of urine. Twenty-four hour urine was collected and placed in pre-labeled polypropylene vials. Furthermore, a single dose-group urine pool composed of 200 µL of urine from each animal in the group was created and stored in a separate vial. Urine vials were placed in cryoboxes and frozen at −20 °C, then shipped on dry ice to the National Center for Environmental Health (NCEH) laboratory at the Centers for Disease Control and Prevention (CDC). Upon arrival, the samples were stored at −70 °C until analyzed. Only pooled urine samples from the first experiment were available for analysis; individual and pooled urine samples were available for the second experiment.

Serum collection

The serum samples were collected only for the second experiment. After sacrifice, whole blood (5 ml) was collected from the inferior vena cava through a 5 ml glass syringe, placed in a tube without anticlotting agents, and left at room temperature for 30 minutes. The clot was removed by centrifugation at 2,500 x g for 10 minutes. The serum (supernatant) was transferred into a clean polypropylene vial using a glass Pasteur pipette. The serum samples were placed in a cryobox and frozen at −20 °C, then shipped on dry ice to the CDC's NCEH laboratory. Upon arrival, the samples were stored at −70 °C until analysis.

Laboratory analysis

Urine and serum samples were analyzed at the CDC's NCEH laboratories for MEP, the common biomarker of DEP, triclosan and MPB. MEP, the specific monoester metabolite of DEP, is measured as a biomarker of exposure to DEP because the measurement of the parent compound poses several challenges (Koch and Calafat 2009). Phthalate diesters are ubiquitous in the environment, can be detected in the laboratory setting, and are quickly metabolized into their hydrolytic monoester (Koch and Calafat 2009). Therefore, MEP is the preferred biomarker because it is not as prone to contamination (Koch and Calafat 2009). Analytical methods for these three biomarkers have been published (Silva et al. 2007a;Ye et al. 2005;Ye et al. 2008). Conjugated species of the biomarkers were enzymatically hydrolyzed, pre-concentrated by on-line solid phase extraction, and separated from other matrix components by high performance liquid chromatography. Quantitation was achieved by isotope dilution tandem mass spectrometry. Limits of detection (LOD), calculated as three times of the standard deviation as the concentration approaches zero (Taylor 1987) for MEP, MPB and triclosan were 0.6, 1.0 , 2.3 ng/mL in urine, and 0.6 , 0.1, 1.1 ng/mL in serum, respectively. Standards, quality control samples, and reagent blanks were included in each analytical batch along with the animal samples. Quality control samples were evaluated according to standard statistical probability rules (Caudill et al. 2008).

Statistical methods

For the second experiment where individual urine and serum samples were available, we examined the associations between oral dose and individual urinary/serum biomarker concentration using linear regression models. Values below the LOD were imputed as LOD/√ 2. We also examined the correlation between urinary and serum biomarker concentrations using Spearman correlation coefficients. Serum MEP concentrations in samples from controls treated with olive oil (mean 136.2 ± 37.8 ng/mL) were greater than serum concentrations measured in samples from animals with the lowest DEP dose (mean 15.8 ± 8.9 ng/mL), indicating a potential contamination problem.. Investigation of both the animal experiment protocol and the analytic laboratory protocol did not reveal any obvious reasons for these results. To avoid sacrificing additional animals, a decision was made to use MEP concentration in serum from the 10 rats in the lowest dose categories of the triclosan and MPB groups as the control concentrations in the regression analyses for the association between oral DEP and serum MEP. Statistical analyses were performed using SAS 9.3 for Windows (Cary. NC. USA).

Biomarker concentrations measured in the rat's urine were compared to the 2009-2010 National Health and Nutrition Examination Survey urine concentration median and 95th percentile (ng/mL) for females. These concentrations were 59.6 and 988 (MEP); 106 and 1230 (MPB); 10.5 and 488 (triclosan) (Centers for Disease Control and Prevention 2014).

Results

Associations between urinary metabolite concentration and oral dose

For the first experiment, only a single pooled urine sample for each experimental group (5 rats) was analyzed for MEP, MPB or triclosan. The results are presented in Table 2. The underlying goal of these experiments was to find oral doses of the three chemicals that resulted in urinary biomarker concentrations in the range reported among the US female population. All dose groups in the first experiment had concentrations far exceeding the 95th percentile (100-times) and the geometric mean (1000-times) of those reported in NHANES (CDC 2014). Therefore, we conducted a second experiment using much lower doses: NOAEL/200, NOAEL/10,000, and NOAEL/100,000 for DEP and MPB, and NOAEL/200, NOAEL/1,000, and NOAEL/10,000 for triclosan. In contrast to the first set of experiments, individual urine samples from the 5 rats in each treatment group were analyzed. Figure 1 presents the mean (± standard deviation) for urinary concentrations of MEP, MPB, and triclosan for each dose group. For all three chemicals, the mean urinary concentrations for the lower oral doses were in the range of urinary concentrations reported for the U.S. female general population (CDC 2014). As shown in Table 3, the urinary concentrations demonstrated a strong linear relationship with the oral doses for each of the three chemicals. Moreover, all R^2 were greater than 0.80 ($P < 0.001$).

Associations between metabolite serum concentration and oral dose

We examined the association between serum concentration and oral dose of the three chemicals in the second experiment. While MEP was detected in all serum samples, MPB was detected in 60% (Table 2). Triclosan was detected in the serum of all dosed animals while all control animals had undetectable serum triclosan concentrations. In general, the concentrations of MEP, MPB, and triclosan in urine were considerably higher than those in serum, ~10^5 times for MEP, ~10^4 times for MPB and ~10 times for triclosan. Spearman correlation coefficients between urine and serum concentrations were 0.44 (MEP), 0.23 (MPB), and 0.98 (triclosan). As shown in Table 3, a relationship between oral dose and biological concentration for MEP was present in urine only (R^2: 0.84 in urine and 0.04 in serum). For MPB, the relationship existed in both urine and serum, but stronger in urine (R^2: 0.85 in urine and 0.57 in serum). The decreased strength in association for serum may be due to the higher detection frequency in the controls compared to the two lower oral doses. For triclosan, the relationship was equally strong in both fluids (R^2: 0.88 in urine and 0.88 in serum).

Discussion

We carried out a systematic dosing study in a rodent model; we identified oral doses of three commonly-used personal care product ingredients that result in comparable urinary biomarker concentrations observed in the female US population. Results of this study may provide important information for future risk assessments of these chemicals that are more reliable in translating to human populations.

The true daily intake of these three chemicals in humans is unknown, however there have been several efforts made at estimating intake and the oral doses used in experiment 2 compare well. For example, the NOAEL/100,000 experimental oral dose for both DEP and MBP (17.4 and 10.5 µg/kg/day, respectively) were only one order of magnitude higher than the estimated median (95th percentile in µg/kg/day) daily intake estimates for DEP, 1.7 (25) in the general female Canadian population aged 20-39 (Saravanabhavan et al. 2014) and MPB, 0.13 (0.36) in the general adult female Chinese population (Liao et al. 2013). The lowest oral dose of triclosan (NOAEL/10,000 = 0.5 µg/kg/day) was within the range of the estimated median (90th percentile in µg/kg/day) daily intake among an adult Belgian study population (0.017 (0.565)) (Geens et al. 2015). Estimation of daily intake depends on knowledge of the toxicokinetics, including administration, distribuion, metabolism and excretion (Soeborg et al. 2014), which were not all necessarily available to the cited authors for performing these calculations. However, the doses used in the second round of experiments do fall within the range of these estimates calculated from adult human populations and provide support for the use of these oral doses in future translational animal experiments.

When conducting exposure studies with low doses, contamination can be a concern especially when the chemicals under study are ubiquitous in the environment. Careful protocols were implemented in the animal laboratory to minimize as much as possible all potential sources of contamination, including: staff avoiding the wearing of perfume and other scented products; testing of the olive oil (vehicle) for the presence of the three chemicals; the use of glass storage containers and glass syringes for all chemicals and solutions; and cleaning procedures that used only hot water without detergent. Although highly unlikely due to the design of the metabolic cages used to collect the urine samples, fecal contamination of the urine could have occurred. Similar strict protocols to avoid external contamination with the target biomarkers were also implemented in the analytic laboratory (Ye et al. 2013).

The detection of biomarker trace concentrations (i.e., parts-per-billion) in all of the control urine samples may suggest a low endemic environmental exposure of the three chemicals that could not be controlled even with the measures undertaken. However, the magnitude of the possible contamination was far below the measured concentrations in urine from rats that received the majority of the experimental doses. Measured urine and serum concentrations likely resulted from both the administered dose plus the low endemic environmental dose. Future studies to repeat or extend the approaches presented here would benefit from use of stable-isotope labeled chemicals in the dosing solution so that measurement of the biomarkers can be confidently attributed to the administered chemical exposure.. To further investigate the unexpected detection of MEP in the 5 control rats serum (see Supplemental Material, Table S1 for individual animal results), samples from animals in the lowest dose group of the other two chemicals (MPB and triclosan) were analyzed for MEP. The assumption was that these rats should be suitable as controls if no MEP contamination occurred. MEP trace concentrations were similar in these 10 rats and slightly lower than the serum concentrations of the group of rats exposed to the lowest DEP dose.

This study was designed to be a dose-calibration investigation. This type of study is normally small in comparison to a main experiment and therefore can provide only limited information on the sources and magnitude of variation of response measures. The sample size was in line with the number of animals per dose group suggested by the Organization for Economic Cooperation and Development (OECD) guidelines (OECD 2010)for toxicokinetic studies. The variability observed among the identically dosed rats is expected due to inter-individual differences in absorption, distribution, metabolism, and excretion of the target analytes, as well as to differences in water intake of the animals during the experiment; the same as would be expected in humans. Although same oral dose (NOAEL/200) was included in experiment 1 and experiment 2, the differences in urinary concentrations in the two experiments likely reflect the normal variability within the animals, and that the experiments were performed at different times, and the animals were of different ages at the start of each experiment. Thus the two experiments should be considered individually. The variability in concentrations of these metabolites among dosed animals has been observed before in SD rats dosed with dibutyl phthalate and di(2-ethylhexyl) phthalate (Calafat et al. 2006;Silva et al. 2007b), other phthalates (Silva et al. 2011), and other non-persistent chemicals, specifically phthalate alternatives (Silva et al. 2012).

Gavage is preferred over other routes of exposure for environmental chemicals when very low doses are used (Vandenberg et al. 2014). It is difficult to ascertain the true intake when chemicals are mixed into food or drinking water ad libitum. Although gavage does not perfectly represent a model of human dietary exposure, this route has been employed for numerous studies assessing potential carcinogenic hazards (Perera et al. 1989).

Animal models are important tools for risk assessment of toxic chemicals and for identifying their potential physiologic consequences. Given the uncertainty of dose extrapolation, it is ideal to carry out risk assessment using exposure conditions that mimic human experience. We chose SD rat as our model system because it has been shown to be one of the most physiologically relevant and genetically defined animal models for studying human sporadic breast cancer (Maltoni et al. 1996;Maltoni et al. 1997). The SD rat-model from RI colony could be considered a human equivalent model, especially for breast lesions (non neoplastic, pre-neoplastic and neoplastic) that will allow us to translate rodent data to humans in future research (Teitelbaum et al. 2014). Furthermore, endocrine effects, which may occur even at the lowest doses, are difficult to detect without a highly sensitive experimental model. SD rats are extremely sensitive and are also recommended by the Endocrine Disruptor Screening Program of the U.S. Enviromental Protection Agency (EPA) which considers them particularly appropriate and relevant for identifying, extrapolating and predicting likely effects in humans (U.S. EPA 2009). In a review of the literature, we found that the range of oral doses for DEP, MPB and triclosan used in animal experiments was wide and much greater in almost all cases than the doses used in our exposure study (see Supplemental Material, Table S2 for listing of these studies). For example, in an investigation of triclosan exposure administered by oral gavage from postnatal day (PND) 19 to 21 and estrogen-dependent responses in rats, the oral dose ranged from 1.18 to 300 mg/kg/day (Stoker et al. 2010). One study of MPB exposure administered by oral gavage from PND 21 to 40 and estrogenic effects used oral doses from 62.5

to 1000 mg/kg/day (Vo et al. 2010). In another investigation of DEP exposure on endocrine-mediated properties, the chemical was administered by oral gavage for 28 days and the doses were 40 to 1000 mg/kg/day (Shiraishi et al. 2006). In most cases, the lowest doses far exceeded the highest doses (0.25 mg/kg/day for triclosan, 5.25 mg/kg/day for MPB, 8.675 mg/kg/day for DEP) used in this current study. Importantly, only the lowest doses in our study, 500 – 5000 times lower than the highest dose, resulted in biological levels that are comparable to those reported by NHANES. Therefore, most published studies employ doses that are not likely to be representative of exposures experienced by the US population and are likely to be orders of magnitude higher.

NOAEL denotes the highest level of exposure of an organism, found by experiment or observation, at which there is no biologically or statistically significant increase in the frequency or severity of any adverse effects when compared to a control group (Lewerenz 1991). In the present study, we had to choose administered oral doses that were many orders of magnitude lower than the NOAEL to observe urinary metabolite concentrations that were within the range of US population based on NHANES 2009-2010 (CDC 2014). As noted above, the potential low endemic environmental exposure must be considered when evaluating the oral administered dose. This highlights the importance of identifying doses that are a realistic representation of human exposure to these chemicals to aid in the design of future animal experiments using these chemicals.

Study results indicate that oral exposure to three commonly-used environmental chemicals, i.e. DEP, MPB and triclosan, have a strong linear relationship with the urinary concentrations of their corresponding biomarkers, but not as uniformly strong in serum. The relatively weak association between administered oral gavage dose of DEP and MBP and their biomarkers serum concentrations is an example of the growing concern about the use of the proper biologic matrix for exposure assessment (Calafat et al. 2013). The timing of urine and serum collection was the same, i.e. 24 hours after completion of treatment; however the urine was collected over a 24-hour period and the blood was collected as a spot sample. The half-lives of these chemicals are relatively short, so that the interval between exposure and biomarker measurement may influence the findings if half-lives differ according to biologic matrix. Moreover, in general the concentrations of MEP, MPB, and triclosan were much lower in the serum compared to those measured in urine, which is in accordance with human studies (Frederiksen et al. 2010). Taken together, the positive linear oral dose-urinary biomarker associations, the higher percent detection of urinary compared to serum biomarkers, and the relatively low concentration of the biomarkers in serum, all provide support for the use of urine as the appropriate biologic matrix for assessing exposure to these non-persistent chemicals.

Conclusion

This study has identified a range of oral doses of three common environmental chemicals that result in a range of urinary biomarker concentrations in a rat model that are consistent with the range of biomarker concentrations measured in the female US population. Although endemic environmental exposures to the parent chemicals may have contributed to the urine concentrations measured in the rodents, the results of this study highlight the importance of careful consideration of the oral dose used in animal experiments and provide useful information for selecting doses for future studies of DEP, MPB and triclosan that may evaluate their biological effects in an experimental setting.

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Table 1. Administered doses and biological samples available.

	Administered Dose[a]			**Samples[b]**	
Compounds	**Reference**	**mg/Kg/day**	**Rats (N)**	**Urine**	**Serum**
Experiment #1					
Diethyl phthalate	NOAEL/10	173.5	5	Yes	No
	NOAEL/100	17.35	5	Yes	No
	NOAEL/200	8.68	5	Yes	No
Methyl paraben	NOAEL/10	105	5	Yes	No
	NOAEL/100	10.5	5	Yes	No
	NOAEL/200	5.25	5	Yes	No
Triclosan	NOAEL/10	5	5	Yes	No
	NOAEL/100	0.5	5	Yes	No
	NOAEL/200	0.25	5	Yes	No
Control	Olive oil	-	5	Yes	No
Experiment #2					
Diethyl phthalate	NOAEL/200	8.675	5	Yes	Yes
	NOAEL/10,000	0.1735	5	Yes	Yes
	NOAEL/100,000	0.01735	5	Yes	Yes
Methyl paraben	NOAEL/200	5.25	5	Yes	Yes
	NOAEL/10,000	0.105	5	Yes	Yes
	NOAEL/100,000	0.0105	5	Yes	Yes
Triclosan	NOAEL/200	0.25	5	Yes	Yes
	NOAEL/1,000	0.05	5	Yes	Yes
	NOAEL/10,000	0.005	5	Yes	Yes
Control	Olive oil	-	5	Yes	Yes

NOAEL: No Observed Adverse Effect Level.

[a]NOAEL in mg/kg/day for diethyl phthalate =1735; methyl paraben=1050; triclosan=50 mg/kg/day

[b]Only pooled urine samples available in experiment #1; individual urine and serum samples available in experiment #2.

Table 2. Urinary and serum biomarker concentrations by administered oral doses of diethyl phthalate, methyl paraben and triclosan.

	Experiment #1			Experiment #2				
Biomarker	**Oral Dose NOAEL/X**	**Oral Dose mg/Kg/day**	**Urine concentration (ng/mL), pooled sample [a]**	**Oral Dose NOAEL/X**	**Oral Dose mg/Kg/day**	**Urine concentration (ng/mL) Mean ± SD [b]**	**Serum N >LOD / total samples**	**Serum concentration (ng/mL) Mean ± SD**
Monoethyl Phthalate (MEP) [c]								
	10	173.5	2.02×10^6	200	8.68	$1.64 \times 10^5 \pm 6.97 \times 10^4$	5 / 5	19.12 ± 7.56
	100	17.35	2.43×10^5	10,000	0.174	$5.53 \times 10^3 \pm 2.87 \times 10^3$	5 / 5	21.66 ± 9.21
	200	8.68	2.28×10^5	100,000	0.0174	287 ± 53.12	5 / 5	15.76 ± 8.89
		0 [d]	3220		0 [e]	128 ± 82.35	10 / 10 [f]	12.26 ± 2.37
Methyl Paraben (MPB)								
	10	105	5.00×10^5	200	5.25	$4.32 \times 10^4 \pm 1.74 \times 10^4$	5 / 5	1.64 ± 0.48
	100	10.5	9.02×10^4	10,000	0.105	$1.39 \times 10^3 \pm 7.09 \times 10^2$	1 / 5	0.4 [g]
	200	5.25	7.70×10^4	100,000	0.0105	$1.44 \times 10^2 \pm 97.15$	1 / 5	0.4 [g]
		0 [d]	3.85		0 [e]	5.46 ± 2.27	5 / 5	0.96 ± 0.44
Triclosan (TCS)								
	10	5	7910	200	0.25	1692 ± 519.15	5 / 5	186 ± 40.69
	100	0.5	1275	1,000	0.05	288.4 ± 90.15	5 / 5	46.88 ± 20.76
	200	0.25	593.5	10,000	0.005	52.24 ± 18.61	5 / 5	4.72 ± 2.20
		0 [d]	5.4		0 [e]	18.14 ± 12.73	0 / 5	--

NOAEL-No Observable Adverse Effect Level; LOD-Limit of detection

[a] Biomarker concentration measured in a single sample from a urine pool of five individual rats for each dose group

[b] Mean of biomarker concentrations measured in five individual urine samples for each dose group. All biomarkers measured in urine samples were detectable (>LOD); LOD (ng/mL) in urine for MEP, MPB and TCS were 0.6, 1.0, 2.3, respectively

For comparison, the 2009-2010 National Health and Nutrition Examination Survey urine concentration median and 95th percentile (ng/mL) for females were 59.6 and 988 (MEP); 106 and 1230 (MPB); 10.5 and 488 (triclosan) (Centers for Disease Control and Prevention 2014)

[c] Monoethyl phthalate is used as the biomarker of exposure to diethyl phthalate

[d] One control group of 5 rats were used for experiment #1, the urine pool sample was analyzed for all three biomarkers, MEP, MPB and triclosan

[e] One control group of 5 rats were used for experiment #2, the 5 individual urine sample was analyzed for all three biomarkers, MEP, MPB and triclosan

[f] MEP serum controls: MEP concentrations measured in serum from 5 MPB and 5 TCS animals treated with the lowest oral dose (MPB: NOAEL/100,000; TCS: NOAEL/10,000); see methods for details

[g] Concentration measured in the single serum sample among dose group with measurement >LOD; LOD (ng/mL) in serum for MEP, MPB and triclosan were 0.6 , 0.1, 1.1, respectively

Table 3. Relationship of urinary and serum biomarker metabolites with oral administered dose of di-ethyl phthalate, methyl paraben and triclosan.

Exposure (Oral dose)	**Outcome (metabolite)**	**R^2**	
		Urine	**Serum**
DEP	MEP	0.84	0.04
MPB	MPB	0.85	0.57
TCS	TCS	0.88	0.88

MEP, mono-ethyl phthalate; DEP, di-ethyl phthalate; MPB, Methyl paraben; TCS, triclosan.

Figure Legend

Figure 1. Abbreviations: DEP-diethyl phthalate; MPB-methyl paraben; TCS-triclosan. For oral doses of DEP, mono-ethyl phthalate (MEP) is measured as the biomarker and presented as the urinary metabolite concentration. NHANES - National Health and Nutrition Examination Survey; Data represent the 50^{th}, 75^{th}, and 90^{th} percentiles of urine concentrations (in ng/mL) for the Female U.S. population (> 6 years old) from NHANES (2009-2010). All other data represents the mean +/- sd for urine concentrations in experimental animals

Figure 1. Urinary metabolite concentration of mono-ethyl phthalate, methyl paraben and triclosan by oral dose (N=5 rats per dose group) and in NHANES (2009-2010)